

1 Title

2 **Independent validation of body fluid-specific CpG markers and construction**
3 **of a robust multiplex assay**

4
5 Authors

6 Hannah Holtkötter*

7 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

8 hannah.holtkoetter@ukmuenster.de

9
10 Vanessa Beyer

11 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

12 vanessa.beyer@gmx.de

13
14 Kristina Schwender

15 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

16 kristina.schwender@ukmuenster.de

17
18 Alina Glaub

19 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

20 alina.glaub@ukmuenster.de

21
22 Kristina Schulze Johann

23 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

24 kristina.schulzejohann@ukmuenster.de

25
26 Marianne Schürenkamp

27 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

28 marianne.schuerenkamp@ukmuenster.de

29
30 Ursula Sibbing

31 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

32 ursula.sibbing@ukmuenster.de

33
34 Sabrina Banken

35 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

36 sabrina.banken@ukmuenster.de

37
38 Peter Wiegand

39 Institute of Legal Medicine, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany

40 Peter.Wiegand@uniklinik-ulm.de

41
42 Heidi Pfeiffer

43 Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.

44 heidi.pfeiffer@ukmuenster.de

45
46 Lynn Dennany

47 Technology and Innovation Centre, Dept. of Pure & Applied Chemistry, University of Strathclyde, 99

48 George Street, Glasgow G1 1RD, Scotland, UK

49 lynn.dennany@strath.ac.uk

50
51 EUROFORGEN-NoE Consortium

Marielle Vennemann
Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany.
marielle.vennemann@ukmuenster.de

*Corresponding author

Highlights

- A set of only four highly tissue specific tDMR markers is sufficient for reliable and robust identification of semen, saliva, venous blood, and menstrual blood.
- A multiplex assay was developed for simultaneous detection of these fluids.
- The multiplex assay can be applied to single source fluids as well as mixtures.
- The components of mock crime scene stains were successfully identified.

Keywords

body fluids; epigenetic markers; DNA methylation; multiplex

Abstract

Potential forensic use of tissue-specific DNA methylation markers has recently been discussed for the identification of the biological source of a stain. In this study 13 promising markers were evaluated to identify suitable candidate markers for the development of a robust and reliable multiplex assay. The results of this study suggest that a combination of only four highly informative markers will be enough for clear body fluid identification. A multiplex assay was developed for the identification of menstrual blood, saliva, semen, and venous blood. This assay was successfully applied to the identification of these body fluids in mixtures and crime scene stains. The multiplex assay aids in the identification of not only single source body fluids but also of body fluid mixtures. The main advantage of using DNA methylation assays over alternative tests is that it can be applied at a later time point in the investigative process since testing is possible even after DNA analysis.

1. Introduction

Identifying the biological source of a crime scene stain can be crucial for the course and outcome of police investigations, and aid in crime scene reconstruction. Due to the importance of body fluid identification in forensic settings, various methods have been developed and improved for this purpose over the last decades [1]. Many such tests rely on catalytic, enzymatic or immunological activities [2], and have been used successfully in identifying venous blood [3], saliva, semen [4], and menstrual blood [5,6]. They are, however, presumptive in nature, and designed to detect one specific body fluid, requiring the investigator to perform a test based on the fluid that is most likely to be present. An alternative approach is the analysis of tissue-specific messenger RNA (mRNA) [7,8] and microRNA [9] as these assays do not cross react with non-biological materials and allow for simultaneous testing of several body fluids [10]. Yet RNA-based assays suffer from the fact that RNA is less stable than DNA and require additional consumption of samples, unless the RNA is co-extracted with DNA.

Recently, potential forensic use of DNA epigenetic markers has been discussed for the identification of the biological source of a stain [8,11–13]. The term epigenetics refers to regulatory mechanisms of the genome that, in contrast to genetic variations, control cell dynamics and gene regulation without modifying the sequence of the DNA. One of several epigenetic mechanisms that cells use to control gene expression is DNA methylation. DNA methylation is the chemical modification of the DNA in which a methyl group (-CH₃) is added to the 5' position of the pyrimidine ring of cytosines in CpG dinucleotides [14], and is generally understood as an inhibitor of DNA transcription [15]. The theory that DNA methylation patterns may be used for the identification of various body fluids is based on the finding that the expression of genes can be tissue specific. DNA methylation is fundamental in the control of cell growth and cellular differentiation and thus, different cell types are specifically fingerprinted through their methylation profile. These methylation profiles believed to be tissue-specific are called „tissue-specific differentially methylated regions“ (tDMRs). The potential use of tDMRs for the identification of body fluids has been indicated by recent advances in whole genome epigenetic analyses and current research in forensic science suggested the use of tDMRs within the human genome as possible marker for human specific body fluid identification [8,16].

Methylation assays show several advantages over conventional presumptive tests such as high sensitivity and specificity to avoid cross-reactivity with other species or materials. Moreover, DNA as a target is particularly appealing as DNA is more stable than RNA, allowing cell identification even in highly degraded or aged samples. DNA methylation assays are also compatible with other DNA-based methods that are commonly used in forensic analyses such as DNA extraction, polymerase-chain-reaction (PCR), and the generation of a DNA profile [8]. Additionally, the development of a multiplex detection assay allows for simultaneous detection of various body fluids while conventional presumptive tests target only one tissue type at a time and require the analyst to make previous assumptions regarding the type of tissue present in a trace.

It is to be expected that methylation analysis will soon become an important tool for the forensic investigation of crime, and therefore, it is essential to develop appropriate detection assays that allow the fast, robust and ultrasensitive detection of differentially methylated DNA sequences.

In our study 13 markers were evaluated in singleplex assays during initial testing to identify suitable candidate markers for the identification of human body fluids. A minimum of distinctive markers was then combined to develop a robust and reliable multiplex assay, with as few as possible markers. Analyzed body fluids were menstrual blood from days 1, 2, and 3 of the menses, saliva, semen, and venous blood.

2. Materials and Methods

2.1 Selection of appropriate tDMRs for body fluid identification

A literature review was performed to identify the most promising candidate markers for the identification of semen, saliva, venous blood, and menstrual blood. Main criterion for selection was the tissue specificity of methylation states. A total of 13 candidate markers were selected for initial testing: Four for the detection

of menstrual blood [17–20], one for the detection of saliva [20], six for the detection of semen [17–20], and two for the detection of venous blood [19,20] (Table S1). Menstrual blood markers were intended to identify vaginal fluid in the original studies [17–20] and tested for their applicability to detect menstrual blood in this study.

2.2 Sample collection and preparation

Body fluid samples were collected from 43 volunteers (16 females and 27 males) with informed consent using procedures approved by the local ethical committee (Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster). Venous blood (n = 13) was collected by venipuncture, and semen (n = 17) was collected in plastic cups. Both fluids were stored frozen in 200 µl aliquots. Menstrual blood was obtained using sterile cotton swabs on the first three days of the menses (n = 10 for each day), dried, and stored at room temperature. Saliva (n = 18) was collected by cheek swabs using sterile cotton swabs and by discharging saliva into a tube. The cotton swabs were dried, and stored at room temperature, and liquid saliva was stored frozen in 200 µl aliquots. For the initial investigation of the markers in singleplex nine menstrual blood samples, ten saliva samples, six semen samples, and ten venous blood samples were used. The sample set was increased from 9 to 30 menstrual blood, 10 to 18 saliva, 6 to 17 semen, and 10 to 13 venous blood samples for the development of the final multiplex assay.

DNA was extracted using the DNA IQ™ Casework Pro Kit on the Maxwell® 16 Forensic Instrument (Promega, Mannheim, Germany). Half a cotton swab per menstrual blood and saliva sample, and 3 µl of each venous blood and semen sample were extracted in 350 µl of Casework Extraction Buffer that was prepped with 19 µl of Proteinase K (Casework Extraction Kit, Promega). Samples were incubated for 1h at 70°C and subsequently lysed in 200 µl lysis buffer. DNA was eluted in 50 µl elution buffer, and quantified using Promega's PowerQuant® System with 1 µl sample, 5 µl 2X Master Mix, 0.5 µl 20X Primer/Probe/IPC Mix, and HPLC-grade water to a reaction volume of 10 µl. DNA concentration of the samples was adjusted to the optimal DNA input for bisulfite treatment of 200 to 500 ng.

2.3 Bisulfite treatment, PCR, and methylation SNaPshot

Bisulfite treatment was performed using the EZ DNA Methylation™ Kit (Zymo Research, Freiburg, Germany) according to manufacturer's recommendations. Bisulfite conversion efficiency was calculated by measuring genomic DNA content before and after bisulfite treatment using the PowerQuant® system as described above.

Bisulfite-converted DNA was amplified in a 12.5 µl reaction volume containing 1 µl template DNA, 1.25 µl dNTPs (100 mM), 1.5 µl MgCl₂ (25 mM), 0.5 µl AmpliTaq Gold® DNA Polymerase and 1.25 µl AmpliTaq Gold Buffer 10 x (5U/µl) Thermo Fisher Scientific, Darmstadt, Germany), and 0.2 µl BSA (MoBiTec, Göttingen, Germany). PCR primers for the amplification of bisulfite-converted DNA and single-base extension (SBE) primers for the target CpGs were slightly modified using sequences from previous reports

[17–20] (Tables S2 and S3). PCR primers were designed to amplify bisulfite-treated DNA. All primers were synthesized by and purchased from Biomers (Germany) and diluted in water to reach a concentration of 100 pmol/μl.

Primer reactivity and marker performance were tested in singleplex reactions. Subsequently, a multiplex reaction was developed using the most tissue specific markers.

For the initial testing of all markers, primer concentrations ranged between 0.4 - 8 μM. For the final multiplex PCR assay primer concentrations were optimized to concentrations ranging from 0.16 - 53.3 μM (see supplementary Table S2 and S3 for details). PCR was performed under the following conditions: 96°C for 10 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; followed by a final extension at 60°C for 1 hour. PCR products were purified by adding 0.025 μl of exonuclease I (New England Biolabs, Frankfurt, Germany) and 1.25 μl of rAPid Alkaline Phosphatase (Roche Diagnostics, Mannheim, Germany) to each sample under the following conditions: 37°C for 95 min and 78°C for 15 min. The following SBE reaction was performed using SNaPshot™ technology (Thermo Fisher Scientific): 1 μl of purified PCR products, 0.36 μM of SBE primers, and 1.25 μl of SNaPshot® Ready Reaction Mix, and HPLC-grade water were used to a reaction volume of 5.5 μl under the following conditions: 25 cycles of 96°C for 10 sec, 55°C for 5 sec, and 60°C for 30 sec; and a final extension at 60°C for 10 min. SBE products were purified with 1 μl of rAPid Alkaline Phosphatase under the following conditions: 37°C for 75 min and 78°C for 15 min. To 1 μl of purified SBE product 10 μl of Hi-Di™-Formamide including GeneScan™ 120 LIZ Size Standard (Thermo Fisher Scientific) were added, denatured at 95°C for 3 min, and subsequently analyzed using the 3130 Genetic Analyzer with the GeneMapper® ID software by Thermo Fisher Scientific.

The multiplex assay was further used for a mixture analysis of fluid venous blood, fluid semen, fluid saliva, and fluid menstrual blood from day one of the menses. This was done by combining bisulfite-converted DNA from different body fluids 1:1 to ensure equal DNA concentrations, and analyzing the mixtures using the developed multiplex assay. Additionally, the multiplex assay was tested for its applicability to crime scene stains by creating five mock crime scene stains by combining body fluids with different mixing ratios or as single body fluid to a total volume of up to 12 μl. The mixtures were applied to cellulose swabs and a small piece was cut out and used for DNA extraction and further analyses described above. The stains were prepared by an independent laboratory assistant who was not involved in the final interpretation of their composition. The analyst was given no information of the mixtures' compositions before analyzing the stains using the GeneMapper® ID software.

2.4 Analysis criteria and statistical analysis

As quality control for the bisulfite-conversion reaction, bisulfite treatment of samples with a bisulfite-conversion efficiency <99.5 % was repeated.

Two main criteria were set for the assessment of marker performance in singleplex as well as multiplex assays as follows: Ability to discriminate between body fluids and absence of background noise in the SNaPshot assay. The ideal marker shows “on-/off-methylation” hence hypermethylation in one body fluid and hypomethylation in all other fluids or *vice versa*. Ideally the difference in methylation state should be obvious by visible inspection through analysis of electropherograms rather than being detectable by application of statistical analysis of large sample sets only. As background noise in electropherogram signals might interfere with the clear interpretation of the markers only those with a clear baseline and low to no background noise were included for further investigation.

The relative methylation state (%) of each CpG site was determined by calculating the relative peak height of the methylated cytosine compared to the combined peak height of the methylated and unmethylated peaks. The information of the methylation state of each sample was summarized in form of box-whisker-plots, where the box represents 50 % of the data with the band inside indicating the median. The whiskers each represent 25 % of the data including the maximum and minimum value. Circles represent outliers and asterisks represent extreme values.

Statistically significant differences between the different body fluids were calculated using the Mann–Whitney *U* test. A value of $p < 0.01$ was considered statistically significant, a value of $p < 0.001$ was considered highly statistically significant. The calculations and descriptive statistics were carried out using SPSS version 22.

3. Results and discussion

3.1 Singleplex SNaPshot assay

The 13 selected markers from relevant literature [17–20] were investigated in singleplex PCR and SNaPshot assays. The semen-specific marker SE3 produced mainly unspecific signals in the SNaPshot assay and data was not useable for interpretation (data available on request). All other markers performed well in the initial singleplex testing and were further investigated in a multiplex assay approach. For the initial singleplex as well as multiplex development, menstrual blood from day one of the menses was used.

3.2 Multiplex SNaPshot assay

Figures S1 – S4 (appendix) show relative methylation (%) for all analyzed markers that were further investigated in multiplex assays. Only markers that allowed clear differentiation between body fluids were included in the development of a final multiplex assay:

3.2.1 tDMRs for the detection of menstrual blood

Based on the results of previous reports [17] the tDMR for PFN3 was selected as a menstrual blood-specific marker because of overall lower methylation levels in menstrual blood compared to all other body fluids. In our study, the methylation state for PFN3 was found to be significantly lower in menstrual blood compared to saliva and venous blood ($p < 0.001$), and significantly higher than in semen ($p < 0.01$). Based

on these findings, the methylation state of PFN3 might be regarded as specific for menstrual blood; however, although statistically significant differences were found between menstrual blood and semen, 31 % of the menstrual blood samples expressed a similar methylation state as 50 % of semen samples. Marker PFN3 was therefore excluded from further analyses (Fig.S1A). The tDMR for VF1, with previously reported hypermethylation in menstrual blood [20], expressed significant differences in relative methylation only between menstrual blood and semen and saliva ($p \leq 0.001$). No statistically significant differences in methylation states between menstrual blood and venous blood were detected, thus VF1 could not be validated as menstrual blood-specific and was excluded from further analyses (Fig.S1B). Menstrual blood expressed significant hypermethylation in VF2 compared to semen, saliva, and blood ($p < 0.001$). It was validated as menstrual blood-specific marker confirming previous findings [20] and was added to the final multiplex assay (Fig.S1C). Marker PRMT2 was previously reported as being hypermethylated in menstrual blood [17,18]. Our findings strongly supported this with significantly higher methylation in menstrual blood compared to semen ($p < 0.001$). Relative methylation in menstrual blood was different from relative methylation in saliva and venous blood; however, this finding was not statistically significant. Consequently, this marker was not considered as a menstrual blood-specific marker for the final multiplex (Fig.S1D).

3.2.2 tDMRs for the detection of saliva

Analysis of SA1 confirmed the results of previous studies [20] as only in saliva it showed almost complete methylation, while all other body fluids expressed hypomethylation. Differences in relative methylation were highly significant ($p < 0.001$), allowing to unambiguously distinguish saliva from semen, venous blood, and menstrual blood (Fig.S2). Thus, it was validated successfully as a saliva-specific marker and was added to the final multiplex assay.

3.2.3 tDMRs for the detection of semen

The markers SE1 (Fig.3A) and SE2 (Fig.S3B) were successfully used for the detection of semen as only semen samples expressed methylation ($p < 0.001$). These results support previous findings of on-/off-methylation [19,20] and confirm SE1 and SE2 as semen-specific markers. In our study, SE2 showed lower intersample variation than SE1 and was therefore included in the development of the final multiplex assay. Relative methylation for the tDMR for marker USP49 (Fig.S1C) was significantly lower in semen compared to saliva, menstrual blood, and venous blood ($p < 0.001$). Although USP49 was validated [17,18] as a semen-specific marker statistically, 37 % of the semen samples expressed a similar methylation state as 10% of the venous blood samples, and the marker was not considered for the development of the final multiplex assay. Previously reported hypomethylation in PRMT2 in semen [17,18] could be confirmed in this study. Relative methylation in semen was significantly lower than in menstrual blood, venous blood, and saliva ($p < 0.001$). High intersample variation in relative methylation amongst venous blood samples was observed, with 25 % of semen samples expressing a similar methylation state as 5 % of the venous blood samples (Fig.S3D). Although statistically validated as a semen-specific marker, PRMT2 was not included in further analyses due to the overlapping results. In the tDMR for DACT1 (Fig.S3E) semen

expressed significantly lower relative methylation than all other body fluids ($p < 0.001$) and was successfully validated as marker for semen detection [17,18]. However, marker SE2, showing hypermethylation of semen only, was chosen over DACT1. Due to epigenetic reprogramming, spermatozoa are expected to be hypomethylated [21] making marker SE2 even more interesting for semen identification since it is a rare example of hypermethylation.

3.2.4 tDMRs for the detection of blood

Marker BL1 (Fig.S4A) expressed statistically significant hypermethylation in venous blood with low to no methylation in all other body fluids ($p \leq 0.001$) and supported the previously reported properties as a venous blood-specific marker [20]. While BL2 (Fig.S4B) was expected to be venous blood-specific based on the results of previous reports [20], our study suggests saliva-specificity as only saliva samples expressed complete unmethylation. Relative methylation in venous blood was significantly higher than in saliva ($p < 0.001$), and menstrual blood ($p < 0.01$) but slightly lower than in semen ($p > 0.01$). Although this marker could be validated as blood-specific it was not chosen for the final multiplex as marker BL1 expressed hypermethylation in venous blood only and was therefore considered more reliable.

3.3 Multiplex SNaPshot assay

For the final multiplex assay only markers for which our findings confirmed results of previous studies and allowed clear differentiation between body fluids were considered for the development of the final multiplex assay. We found that only four markers allow complete differentiation between the four body fluids: These markers showed to be the menstrual-blood specific marker VF2, the saliva-specific marker SA1, the semen-specific marker SE2, and the venous blood-specific marker BL1.

Figure 1A shows the developed multiplex SNaPshot assay including the four markers, exemplarily for one sample of each body fluid sample: semen (Fig.1A1), saliva (Fig.1A2), venous blood (Fig.A3), and menstrual blood of the three days of the menses (MB-1, MB-2, MB-3; Fig.1A4 – 1A6). The markers were being detected on the reverse-complementary strand: The blue peak, representing guanine, shows the methylated portion as methylated cytosine remains unchanged in the bisulfite-conversion reaction and binds guanine. The green peak, representing adenine, shows the unmethylated portion as unmethylated cytosine is being converted into uracil in the bisulfite-conversion reaction, amplified as thymine in the PCR and therefore binding adenine. The selection of the four tDMRs resulted in highly specific methylation patterns, which allow immediate visual distinction between semen, saliva, venous blood, and menstrual blood.

Marker VF2 expressed partial to strong methylation in menstrual blood from all three days of the menses and nearly complete lack of methylation in all other body fluids ($p < 0.001$; Fig.2A). Menstrual blood samples showed a clear blue peak in the electropherograms, while saliva, semen, and venous blood samples showed no to very small blue peaks, resulting in a clear visual identification of menstrual blood.

307 Marker SA1 exhibited significantly saliva-specific hypermethylation ($p < 0.001$) with an even higher
308 specificity compared to literature data [21] (Fig.2B). High blue peaks were visible in the electropherograms
309 in saliva samples with complete lack of methylation or blue peaks in semen and menstrual blood samples.

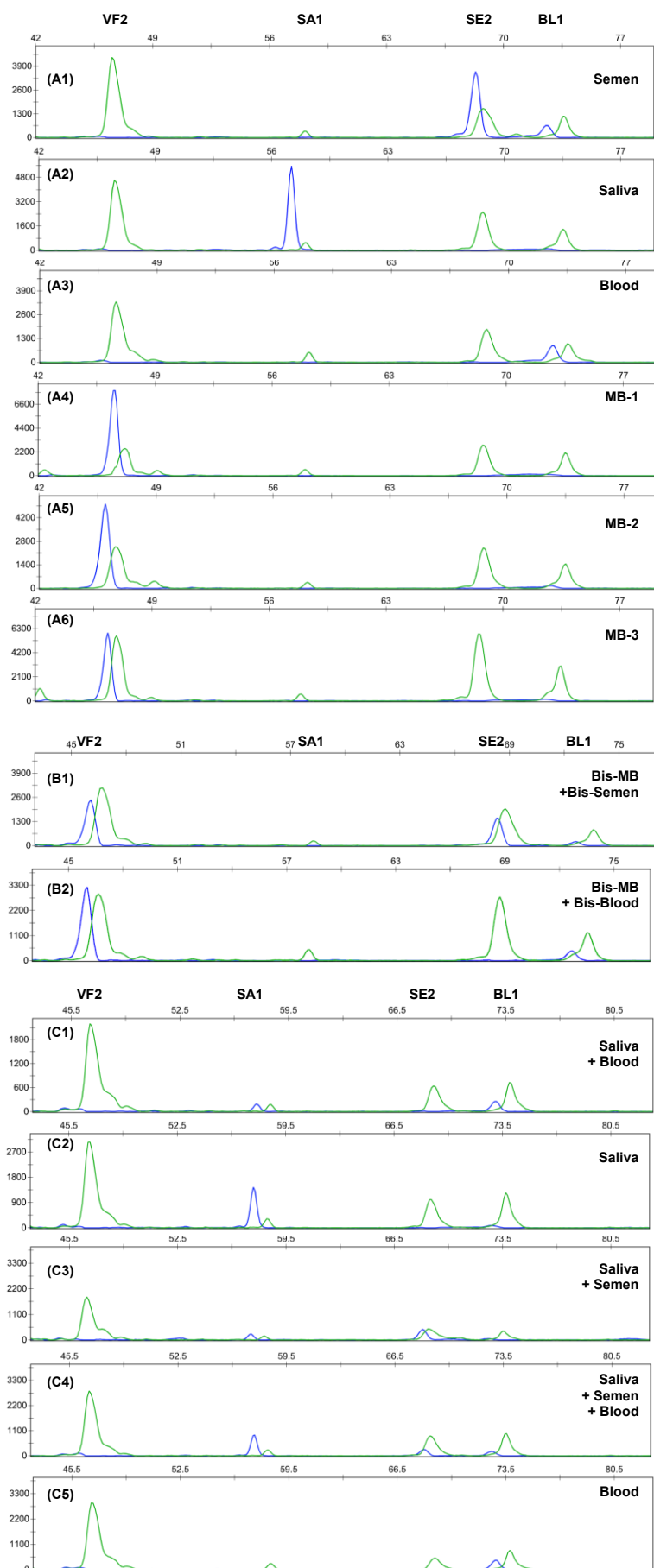


Fig. 1 Representative electropherogram of the multiplex SNaPshot assay. The blue peak (for guanine) shows the methylated and the green peak (for adenine) the unmethylated portion. From left to right: The menstrual blood- specific marker VF2, the saliva-specific marker SA1, the semen-specific marker SE2, and the venous blood-specific marker BL1.

A shows the developed multiplex SNaPshot assay exemplarily for one sample of each body fluid sample: semen (A1), saliva (A2), venous blood (A3), and menstrual blood of the three days of the menses (A4-6). Marker VF2 exhibits menstrual blood-specific, marker SA1 saliva-specific, and marker SE2 semen-specific hypermethylation. Marker BL1 shows a higher methylation state in semen and blood. MB-1, MB-2, MB-3 = menstrual blood of days 1, 2, and 3 of the menses.

B shows 1:1 mixtures of bisulfite-converted DNA. Partial methylation is exhibited in the mixtures of menstrual blood (MB) and semen in the menstrual blood-specific marker VF2 and the semen-specific marker SE2 (B1). Partial methylation is exhibited in the mixtures of menstrual blood and venous blood in the menstrual blood-specific marker VF2 and blood-specific marker BL1 (B2). Bis = Bisulfite-converted DNA sample

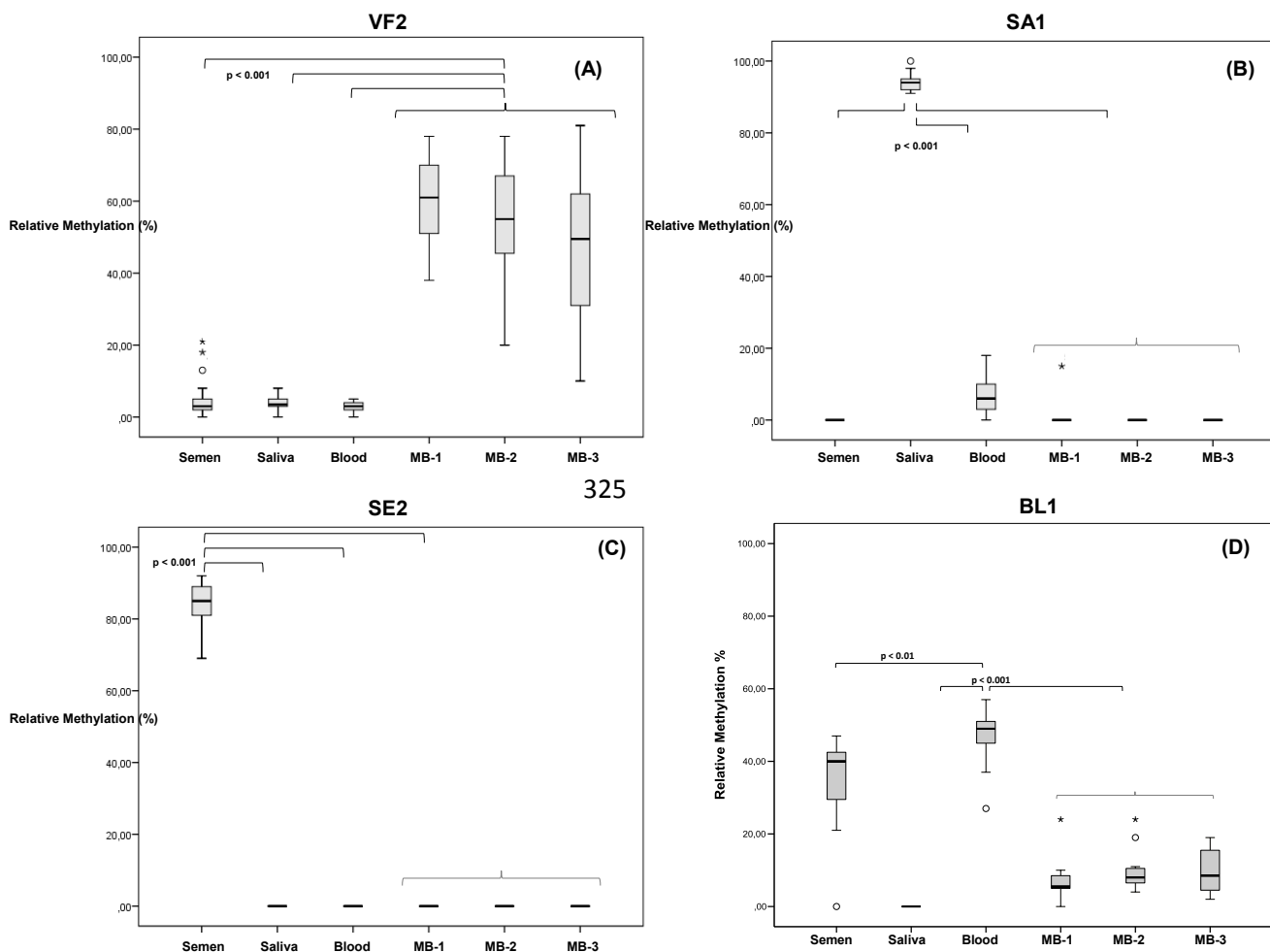
C shows the analysis of the mock crime scene stains. Stain C1 contained venous blood and saliva, stain C2 solely saliva, stain C3 saliva and semen, stain C4 saliva, semen as well as blood, and stain C5 solely venous blood. It was possible for the analyst to correctly determine the composition of the five stains based on analysis of the electropherograms.

311 Although numerous venous blood samples (92 %) also expressed a very low level of methylation, they
 312 were distinguishable by visual comparison of peak height: While saliva samples showed >90%
 313 methylation in all samples analyzed, blood revealed <20% methylation in all samples.

314 Marker SE2 expressed significant semen-specific hypermethylation ($p < 0.001$) with complete lack of
 315 methylation in the remaining body fluids (Fig.2C). Only in semen samples the blue peak, indicating
 316 methylation, was visible while all other body fluids expressed a green peak, indicating no methylation.

317 Marker BL1 showed a significantly higher methylation state in venous blood compared to saliva, menstrual
 318 blood ($p < 0.001$) and semen ($p < 0.01$; Fig.2D). Even though BL1 expresses partial methylation in semen,
 319 a combination of marker BL1 with marker SE2, being hypermethylated in semen and hypomethylated in
 320 venous blood, aids in distinguishing venous blood samples from semen samples.

321 All markers showed to be highly stable with results from the initial multiplex analyses reliably reproduced
 322 in the final multiplex assay. Not only was the difference in relative methylation amongst the different body
 323 fluids statistically significant but also supported the results from the visual analysis of the
 324 electropherograms.



334 **Fig. 2 Relative methylation (%) of the multiplex SNaPshot assay.** Marker VF2 (A) shows a higher methylation state in menstrual blood and nearly complete lack of methylation in all other body fluids ($p < 0.001$). Marker SA1 (B) exhibits saliva-specific, marker SE2 (C) semen-specific hypermethylation ($p < 0.001$). Marker BL1 (D) shows a higher methylation state in venous blood than in saliva, menstrual blood, and semen. MB-1, MB-2, MB-3 = menstrual blood of days 1, 2, and 3 of the menses. Circles represent outliers and asterisks represent extreme values.

3.4 Analysis of body fluid mixtures

Menstrual blood-containing mixtures expressed a significantly higher methylation state in the tDMR for VF2 than mixtures without menstrual blood ($p < 0.001$; Fig.S5A). In SA1 the saliva-containing mixtures expressed a significantly higher methylation state than non-saliva-containing mixtures ($p < 0.001$; Fig.S5B). For the mixtures liquid saliva was used while buccal cells were used for the initial testing phase. The results show that marker SA1 expresses the same methylation profile in liquid saliva and buccal cells. Semen-containing mixtures expressed a significantly higher methylation state in SE2 than non-semen-containing mixtures ($p < 0.001$; Fig.S5C). In BL1 (Fig.S5D) the mixture of venous blood and menstrual blood expressed a significantly higher relative methylation state than the mixtures of semen and saliva ($p < 0.001$), menstrual blood and saliva ($p < 0.01$), and menstrual blood and semen ($p < 0.01$). The mixture of venous blood and semen also expressed a significantly higher methylation state than the mixtures of semen and saliva, menstrual blood and saliva, and menstrual blood and semen ($p < 0.001$). The mixture of venous blood and saliva expressed a significantly higher methylation state than the mixture of semen and saliva ($p < 0.001$). In figure 1B electropherograms are given exemplarily for two mixtures; menstrual blood combined with semen (Fig.1B1), and menstrual blood combined with venous blood (Fig.1B2).

3.5 Analysis of mock crime scene stains

The mock crime scene stains were solely analyzed visually based on electropherograms without the support of descriptive statistics (Fig.1C). It was possible to correctly determine the composition of 5/5 stains. The correct determination included the definition of whether the stain stemmed from a single source or a mixture and then, of which body fluids the mixture was generated as well as the determination of minor and major components within the mixture. Stain C1 contained 2 μ l saliva and 10 μ l venous blood. The saliva-specific marker SA1 and the venous blood-specific marker BL1 both expressed partial methylation with an overall stronger signal in BL1. The menstrual blood-specific marker VF2 as well as the semen-specific marker SE2 were both hypomethylated. Based on this information it was accurately determined that the major component of the mixture was venous blood and the minor component saliva. Stain C2 consisted of 10 μ l saliva, and only marker SA1 showed a clear signal for hypermethylation with all other markers expressing hypomethylation. Accordingly it was correctly concluded that the stain only consisted of saliva. Stain C3's components were 5 μ l saliva and 5 μ l semen. Markers SA1 and SE2 both expressed partial methylation with an overall equal signal intensity. Hypomethylation was observed in markers VF2 and BL1. The stain's composition was accurately determined to be of equal parts semen and saliva. Stain C4 contained 3 μ l saliva, 3 μ l semen, and 3 μ l venous blood. Markers SA1, SE2, and BL1 all showed to be partially methylated while marker VF2 was hypomethylated. It was therefore determined that the stain consisted of saliva, semen, and venous blood in equal concentrations. Stain C5 contained 10 μ l venous blood. Marker BL1 showed to be partially methylated, while markers VF2, SA1, and SE2 expressed hypomethylation. The stain was concluded to stem from venous blood only.

4. Conclusion

The main advantage of using DNA methylation assays over conventional presumptive tests or RNA based methods is that it can be applied even after DNA testing. In all other test methods, a decision on whether or not body fluid identification is necessary needs to be made by the analyst before a DNA typing result was obtained. Thus, methylation analysis complements conventional presumptive tests because it enables body fluid identification at a later time point in the investigative process. This can be very helpful in cases in which the presence of a mixture of body fluids becomes obvious only after DNA typing. While mRNA analyses were shown to also allow the discrimination of body fluid mixtures [24] the decision for RNA/DNA co-extraction needs to be made before DNA typing results are available.

The results of this study suggest that a combination of only four highly informative markers will be enough for clear body fluid identification. The included markers were validated from previous studies and demonstrated to be robust and reliable in the developed multiplex assay. The multiplex assay aids in the identification of not only single source body fluids but also of body fluid mixtures. Both markers BL1 and VF2 allow for the differentiation between venous and menstrual blood. This is especially important in alleged sexual assault cases as the differentiation between venous and menstrual blood could provide valuable information regarding the issue of consent. VF2, however, was originally designed as marker for the detection of vaginal fluid [20]. To also discriminate between mixtures of vaginal fluid with blood and menstrual blood, further markers with specific methylation states in menstrual blood are needed. Lee et al. very recently published two potential candidate markers [25] that still need independent validation. Marker SE2 was shown to be hypermethylated despite the fact that spermatozoa are expected to be hypomethylated due to epigenetic reprogramming, making marker SE2 even more interesting for semen identification since it is a rare example of hypermethylation. Marker SA1 expressed saliva-specific hypermethylation not only in buccal cell swabs but also in liquid saliva. Since it is more likely to encounter saliva in a forensic setting than buccal cells, this marker can be straightforwardly applied to crime scene stains as shown in this study. One main criteria for marker selection was “on-/off-methylation”, meaning hypermethylation in the tissue in question and hypomethylation in all other fluids or vice versa. This leads to a difference in methylation state that is obvious by visible inspection through analysis of electropherograms rather than being detectable by application of statistical analysis of large sample sets only. As the markers SA1, SE2, and VF2 expressed methylation only in their respective body fluids, marker BL1 showed high methylation levels in venous blood as well as semen samples. Consequently, BL1 should be used in combination with marker SE2 to exclude semen presence. To this date no venous blood marker was discovered that expresses strict on-/off-methylation. Although recent studies identified new markers for potential venous blood identification, those markers also did not express venous blood-specific methylation only [22,23,25], stressing the need for further research towards the discovery of a venous blood marker that allows for unambiguous identification.

Conflict of interest:

none

Acknowledgements:

We thank all donors for their participation and the research group at King's College London for providing us with additional semen samples. This research is part of the *European Forensic Genetics Network of Excellence*, funded by the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 285487. It did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References:

- [1] K. Virkler, I.K. Lednev, Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene, *Forensic Sci. Int.* 188 (2009) 1–17. doi:<http://dx.doi.org/10.1016/j.forsciint.2009.02.013>.
- [2] S. Harbison, R. Fleming, Forensic body fluid identification: state of the art, *Res. Reports Forensic Med. Sci.* Volume 6 (2016) 11. doi:10.2147/RRFMS.S57994.
- [3] S.S. Tobe, N. Watson, N. Nic Daéid, Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA, *J. Forensic Sci.* 52 (2007) 102–109. doi:10.1111/j.1556-4029.2006.00324.x.
- [4] M. Vennemann, G. Scott, L. Curran, F. Bittner, S.S. Tobe, Sensitivity and specificity of presumptive tests for blood, saliva and semen, *Forensic Sci. Med. Pathol.* 10 (2014) 69–75. doi:10.1007/s12024-013-9515-6.
- [5] H. Holtkötter, L. Dierig, M. Schürenkamp, U. Sibbing, H. Pfeiffer, M. Vennemann, Validation of an immunochromatographic D-dimer test to presumptively identify menstrual fluid in forensic exhibits, *Int. J. Legal Med.* 129 (2014) 37–41.
- [6] D.J. Baker, E.A. Grimes, A.J. Hopwood, D-dimer assays for the identification of menstrual blood, *Forensic Sci. Int.* 212 (2011) 210–214. doi:10.1016/j.forsciint.2011.06.013.
- [7] C. Haas, B. Klessner, C. Maake, W. Bär, A. Kratzer, mRNA profiling for body fluid identification by reverse transcription endpoint {PCR} and realtime PCR, *Forensic Sci. Int. Genet.* 3 (2009) 80–88. doi:<http://dx.doi.org/10.1016/j.fsigen.2008.11.003>.
- [8] D. Frumkin, A. Wasserstrom, B. Budowle, A. Davidson, DNA methylation-based forensic tissue identification, *Forensic Sci. Int. Genet.* 5 (2011) 517–524. doi:<http://dx.doi.org/10.1016/j.fsigen.2010.12.001>.
- [9] D. Zubakov, A.W.M. Boersma, Y. Choi, P.F. van Kuijk, E.A.C. Wiemer, M. Kayser, MicroRNA

- markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation, *Int. J. Legal Med.* 124 (2010) 217–226. doi:10.1007/s00414-009-0402-3.
- [10] M. van den Berge, A. Carracedo, I. Gomes, E.A.M. Graham, C. Haas, B. Hjort, P. Hoff-Olsen, O. Maroñas, B. Mevåg, N. Morling, H. Niederstätter, W. Parson, P.M. Schneider, D.S. Court, A. Vidaki, T. Sijen, A collaborative European exercise on mRNA-based body fluid/skin typing and interpretation of {DNA} and {RNA} results, *Forensic Sci. Int. Genet.* 10 (2014) 40–48. doi:http://dx.doi.org/10.1016/j.fsigen.2014.01.006.
- [11] A.H.C. Wong, I.I. Gottesman, A. Petronis, Phenotypic differences in genetically identical organisms: the epigenetic perspective, *Hum. Mol. Genet.* 14 (2005) R11–R18. doi:10.1093/hmg/ddi116 .
- [12] V.K. Rakyan, T.A. Down, N.P. Thorne, P. Flicek, E. Kulesha, S. Gräf, E.M. Tomazou, L. Bäckdahl, N. Johnson, M. Herberth, K.L. Howe, D.K. Jackson, M.M. Miretti, H. Fiegler, J.C. Marioni, E. Birney, T.J.P. Hubbard, N.P. Carter, S. Tavaré, S. Beck, An integrated resource for genome-wide identification and analysis of human tissue-specific differentially methylated regions (tDMRs), *Genome Res.* 18 (2008) 1518–1529. doi:10.1101/gr.077479.108.
- [13] R. Illingworth, A. Kerr, D. DeSousa, H. Jørgensen, P. Ellis, J. Stalker, D. Jackson, C. Clee, R. Plumb, J. Rogers, S. Humphray, T. Cox, C. Langford, A. Bird, A Novel CpG Island Set Identifies Tissue-Specific Methylation at Developmental Gene Loci, *PLOS Biol.* 6 (2008) e22. http://dx.doi.org/10.1371%2Fjournal.pbio.0060022.
- [14] M.M. Suzuki, A. Bird, DNA methylation landscapes: provocative insights from epigenomics, *Nat Rev Genet.* 9 (2008) 465–476. http://dx.doi.org/10.1038/nrg2341.
- [15] K.W.K. Lee, Z. Pausova, Cigarette smoking and DNA methylation, *Front. Genet.* 4 (2013) 132. doi:10.3389/fgene.2013.00132.
- [16] A. Vidaki, B. Daniel, D.S. Court, Forensic DNA methylation profiling—Potential opportunities and challenges, *Forensic Sci. Int. Genet.* 7 (2013) 499–507. doi:http://dx.doi.org/10.1016/j.fsigen.2013.05.004.
- [17] H.Y. Lee, M.J. Park, A. Choi, J.H. An, W.I. Yang, K.-J. Shin, Potential forensic application of DNA methylation profiling to body fluid identification, *Int. J. Legal Med.* 126 (2012) 55–62. doi:10.1007/s00414-011-0569-2.
- [18] J.H. An, A. Choi, K.-J. Shin, W.I. Yang, H.Y. Lee, DNA methylation-specific multiplex assays for body fluid identification, *Int. J. Legal Med.* 127 (2013) 35–43. doi:10.1007/s00414-012-0719-1.
- [19] J.-L. Park, O.-H. Kwon, J.H. Kim, H.-S. Yoo, H.-C. Lee, K.-M. Woo, S.-Y. Kim, S.-H. Lee, Y.S. Kim, Identification of body fluid-specific DNA methylation markers for use in forensic science, *Forensic Sci. Int. Genet.* 13 (2014) 147–153. doi:http://dx.doi.org/10.1016/j.fsigen.2014.07.011.

- [20] H.Y. Lee, J.H. An, S.-E. Jung, Y.N. Oh, E.Y. Lee, A. Choi, W.I. Yang, K.-J. Shin, Genome-wide methylation profiling and a multiplex construction for the identification of body fluids using epigenetic markers, *Forensic Sci. Int. Genet.* 17 (2015) 17–24. doi:http://dx.doi.org/10.1016/j.fsigen.2015.03.002.
- [21] S. Güneş, T. Kulaç, The role of epigenetics in spermatogenesis, *Turkish J. Urol.* 39 (2013) 181–187. doi:10.5152/tud.2013.037.
- [22] A. Vidaki, F. Giangasparo, D. Syndercombe Court, Discovery of potential DNA methylation markers for forensic tissue identification using bisulphite pyrosequencing, *Electrophoresis* (2016) 2767–2779. doi: 10.1002/elps.201600261
- [23] S. Forat, B. Huettel, R. Reinhardt, R. Fimmers, G. Haidl, D. Denschlag, K. Olek, Methylation Markers for the Identification of Body Fluids and Tissues from Forensic Trace Evidence, *PLoS One* (2016) e0147973. doi: 10.1371/journal.pone.0147973
- [24] A.D. Roeder, C. Haas, mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification, *International Journal of Legal Medicine* 127 (2013) 707–721. doi: 10.1007/s00414-012-0794-3.
- [25] H.Y. Lee, S.-E. Jung, E.H. Lee, W. I. Yang, K.-J. Shin, DNA methylation profiling for a confirmatory test for blood, saliva, semen, vaginal fluid and menstrual blood, *Forensic Science International: Genetics* 24 (2016) 75–82. doi.org/10.1016/j.fsigen.2016.06.007.

Appendix A. Supplementary Data: Supplementary data associated with this article can be found, in the online _____ version, _____ at _____.

Table S1 Overview of the 13 markers chosen for singleplex analysis

Body fluid	Target ID ¹	Gene	CpG sites	CpG site ²	Marker name ³	Source
Blood	cg06379435	n/a	5	chr19:3344273	BL1	Lee et al. [20], Park et al. [19]
	cg01543184	MAFG	14	chr17:79881543	BL2	Lee et al. [20]
Saliva	cg09652652	FAM43A	17	chr3:194408847	SA1	Lee et al. [20]
Menstrual fluid	cg09765089-231d	n/a	12	chr7:27291577	VF1	Lee et al. [20]
	cg26079753-7d	n/a	9	chr12:54355535	VF2	Lee et al. [20]
	cg15123573	PFN3	12	chr5:176759712	PFN3	Lee et al. [17], An et al. [18]
	n/a	PRMT2	7	chr21:46905904	PRMT2	Lee et al. [17], An et al. [18]
Semen	cg17610929	ASIC4	15	chr2:220379044	SE1	Lee et al. [20], Park et al. [19]
	cg26763284-138u	PLEC	7	chr8:145018185	SE2	Lee et al. [20]
	cg17621389	n/a	6	chr6:147728290	SE3	Lee et al. [20]
	n/a	PRMT2	7	chr21:46905904	PRMT2	Lee et al. [17], An et al. [18]
	n/a	USP49	19	chr6:41881972	USP49	Lee et al. [17], An et al. [18]
	n/a	DACT1	6	chr14:58182894	DACT1	Lee et al. [17], An et al. [18]

1 The TargetID is unique to the Illumina HumanMethylation 450 K bead array used in the studies by Park et al. and Lee et al. [19,20].

2 The CpG site is indicated by the genomic location by the human reference genome 37 (GRCh37/hg19).

3 The marker name was adopted from the according literature [18-21].

Table S2 PCR primers and single-base extension primers for the initial multiplex testing¹.

Initial Multiplex bisulfite PCR primers				Single-base extension (SNaPshot) primers		
Marker	Sequence (5'>3')	Conc. (μM)	Amplicon size (bp)	Sequence (5'>3')	Conc. (μM)	Amplicon size (bp)
BL1	F: <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>G</u> <u>G</u> <u>T</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>T</u> <u>T</u> <u>A</u> <u>G</u>	1.5	159	R: (TG) ₄₇ <u>C</u> <u>C</u> <u>R</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>T</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u> <u>R</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u>	0.36	118
	R: <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>A</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u> <u>T</u> <u>T</u> <u>A</u> <u>C</u> <u>T</u> <u>C</u> <u>C</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>C</u> <u>C</u>	1.5				
BL2	F: <u>G</u> <u>G</u> <u>A</u> <u>G</u> <u>A</u> <u>G</u> <u>G</u> <u>T</u> <u>A</u> <u>G</u> <u>G</u> <u>T</u> <u>T</u> <u>A</u> <u>G</u> <u>A</u> <u>A</u> <u>G</u> <u>G</u> <u>A</u> <u>A</u> <u>G</u> <u>T</u>	0.8	196	R: (TG) ₁₅ <u>C</u> <u>R</u> <u>A</u> <u>C</u> <u>T</u> <u>C</u> <u>T</u> <u>C</u> <u>R</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>R</u> <u>C</u> <u>T</u> <u>A</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u>	0.36	58
	R: <u>C</u> <u>C</u> <u>C</u> <u>T</u> <u>C</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>A</u>	0.8				
SA1	F: <u>G</u> <u>G</u> <u>G</u> <u>G</u> <u>A</u> <u>T</u> <u>T</u> <u>Y</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>Y</u> <u>G</u> <u>T</u> <u>T</u> <u>A</u> <u>G</u> <u>G</u> <u>T</u>	6.4	153	R: (GT) ₁₃ <u>G</u> <u>C</u> <u>C</u> <u>A</u> <u>C</u> <u>G</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>A</u> <u>C</u> <u>G</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u>	0.36	53
	R: <u>C</u> <u>C</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>C</u> <u>C</u> <u>C</u> <u>C</u> <u>T</u> <u>T</u> <u>C</u> <u>C</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u>	8.0				
SE1	F: <u>I</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>A</u> <u>A</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u> <u>G</u>	0.4	174	R: (TG) ₁₅ <u>C</u> <u>C</u> <u>R</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>C</u> <u>T</u> <u>C</u> <u>C</u> <u>C</u> <u>C</u> <u>A</u> <u>C</u>	0.36	46
	R: <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u> <u>T</u> <u>T</u> <u>C</u> <u>C</u> <u>C</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>C</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u>	0.4				
SE2	F: <u>T</u> <u>G</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>T</u> <u>A</u> <u>G</u> <u>G</u> <u>G</u> <u>A</u> <u>G</u> <u>G</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>A</u> <u>G</u>	0.2	105	R: (TG) ₂₀ <u>C</u> <u>R</u> <u>C</u> <u>R</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u> <u>R</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>C</u> <u>T</u> <u>C</u>	0.36	63
	R: <u>C</u> <u>C</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>C</u> <u>R</u> <u>A</u> <u>T</u> <u>T</u> <u>C</u> <u>C</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u>	0.4				
SE3	F: <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>I</u> <u>A</u> <u>T</u> <u>G</u> <u>A</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u> <u>G</u> <u>A</u> <u>T</u> <u>G</u> <u>G</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u>	2.0	127	R: (TG) ₂₂ <u>A</u> <u>C</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>C</u> <u>R</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u> <u>T</u> <u>T</u> <u>C</u>	0.36	31
	R: <u>A</u> <u>T</u> <u>A</u> <u>C</u> <u>C</u> <u>T</u> <u>A</u> <u>C</u> <u>T</u> <u>C</u> <u>A</u> <u>A</u> <u>T</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>C</u>	2.0				
USP49	F: <u>Y</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>G</u> <u>G</u> <u>T</u> <u>A</u> <u>T</u> <u>G</u> <u>G</u> <u>T</u> <u>Y</u> <u>G</u> <u>G</u> <u>T</u>	1.1	150	R: (A) ₁₀ <u>C</u> <u>R</u> <u>C</u> <u>C</u> <u>R</u> <u>C</u> <u>C</u> <u>A</u> <u>C</u> <u>A</u> <u>C</u> <u>R</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>C</u>	0.36	30
	R: <u>A</u> <u>C</u> <u>A</u> <u>C</u> <u>C</u> <u>C</u> <u>C</u> <u>T</u> <u>C</u> <u>C</u> <u>R</u> <u>C</u> <u>R</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u>	1.1				
DACT1	F: <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>G</u> <u>G</u> <u>G</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>A</u> <u>G</u> <u>A</u> <u>A</u>	1.3	115	F: (A) ₁₅ <u>G</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>A</u> <u>G</u> <u>A</u> <u>A</u> <u>Y</u> <u>G</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u>	0.36	37
	R: <u>T</u> <u>T</u> <u>C</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>C</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> <u>T</u> <u>C</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>C</u> <u>A</u>	1.3				
VF1	F: <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>T</u> <u>A</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>A</u> <u>G</u>	0.6	137	R: (TG) ₅ <u>T</u> <u>C</u> <u>C</u> <u>C</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>R</u> <u>A</u> <u>C</u> <u>R</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>C</u>	0.36	36
	R: <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>R</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>R</u> <u>A</u> <u>C</u> <u>C</u> <u>C</u> <u>R</u> <u>A</u> <u>A</u> <u>C</u>	4.8				
VF2	F: <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>G</u> <u>A</u> <u>G</u> <u>T</u> <u>G</u> <u>T</u> <u>G</u> <u>A</u> <u>G</u> <u>A</u> <u>G</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u> <u>G</u> <u>A</u>	0.4	176	R: (TG) ₉ <u>C</u> <u>R</u> <u>A</u> <u>T</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>C</u> <u>C</u>	0.36	41
	R: <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>T</u> <u>C</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>T</u> <u>C</u> <u>T</u> <u>A</u>	0.4				
PFN3	F: <u>G</u> <u>T</u> <u>A</u> <u>A</u> <u>G</u> <u>T</u> <u>G</u> <u>G</u> <u>A</u> <u>G</u> <u>G</u> <u>A</u> <u>G</u> <u>G</u> <u>A</u> <u>G</u> <u>T</u> <u>T</u> <u>A</u> <u>G</u> <u>T</u>	0.6	147	R: (A) ₂₅ <u>R</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>T</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>R</u> <u>C</u>	0.36	52
	R: <u>T</u> <u>A</u> <u>C</u> <u>A</u> <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>T</u> <u>C</u> <u>C</u> <u>T</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u>	0.6				
PRMT2	F: <u>T</u> <u>A</u> <u>A</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>A</u> <u>A</u> <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>A</u> <u>A</u> <u>G</u> <u>A</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>A</u> <u>G</u>	1.0	145	F: (A) ₂₀ <u>T</u> <u>T</u> <u>G</u> <u>A</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>A</u> <u>G</u> <u>Y</u> <u>G</u> <u>T</u> <u>Y</u> <u>G</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>G</u> <u>A</u> <u>T</u> <u>T</u>	0.36	43
	R: <u>T</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>C</u> <u>C</u> <u>C</u> <u>C</u> <u>A</u> <u>A</u> <u>A</u> <u>A</u> <u>C</u>	1.0				

¹ PCR primers are specifically designed for one strand of the bisulfite-converted DNA. In the bisulfite conversion reaction unmethylated cytosine is being converted into uracil and amplified as thymine in the PCR. The nucleotide T in the forward (F) and A in the reverse (R) sequence obtained upon bisulfite conversion of unmethylated cytosine is underlined. The nucleotides that are located at the sites complementary to methylatable cytosines are indicated in bold as Y or R (wobble bases) for the forward or reverse strand, respectively.

Table S3 Multiplex PCR primers and single-base extension primers used for the final methylation-sensitive multiplex SNaPshot assay¹.

Multiplex bisulfite PCR primers				Single-base extension (SNaPshot) primers		
Marker	Sequence (5'>3')	Conc. (μM)	Amplicon size (bp)	Sequence (5'>3')	Conc. (μM)	Amplicon size (bp)
BL1	F: <u>T</u> T <u>T</u> A <u>T</u> TGGGG <u>T</u> ATTT <u>T</u> ATTGGT <u>T</u> AG	12.5	159	R: (TG) ₂₂ CC <u>R</u> AT <u>A</u> AAACCTCA <u>A</u> ACRT <u>A</u> AAAC	0.36	68
	R: AAAAT <u>A</u> CAACTTACTCCT <u>A</u> AAACACC	12.5				
SA1	F: GGGGA <u>T</u> YGT <u>T</u> YGTTAGGT <u>T</u>	53.3	153	R: (GT) ₁₃ GCCACGA <u>T</u> AA <u>T</u> AA <u>T</u> ACCACGAT <u>A</u> AA <u>A</u> C	0.36	53
	R: CCATTTCCTCCT <u>T</u> CTAAAA	6.6				
SE2	F: GGGGA <u>T</u> YGT <u>T</u> YGTTAGGT <u>T</u>	0.16	105	R: (TG) ₂₀ C <u>R</u> CRT <u>A</u> AC <u>R</u> ACTAT <u>A</u> AA <u>A</u> CCCTC	0.36	63
	R: CCATTTCCTCCT <u>T</u> CTAAAA	0.3				
VF2	F: <u>I</u> GTTGATATGTT <u>T</u> TGAATTATTAAG	0.3	176	R: (TG) ₉ C <u>R</u> ATCAACTACTAT <u>A</u> AA <u>A</u> ACACC	0.36	41
	R: <u>A</u> TAACTTCCCTTATCAACACCA <u>A</u> C	0.3				

¹ PCR primers are specifically designed for one strand of the bisulfite-converted DNA. In the

bisulfite conversion reaction unmethylated cytosine is being converted into uracil and amplified as thymine in the PCR. The nucleotide T in the forward (F) and A in the reverse (R) sequence obtained upon bisulfite conversion of unmethylated cytosine is underlined. The nucleotides that are located at the sites complementary to methylatable cytosines are indicated in bold as Y or R (wobble bases) for the forward or reverse strand, respectively.

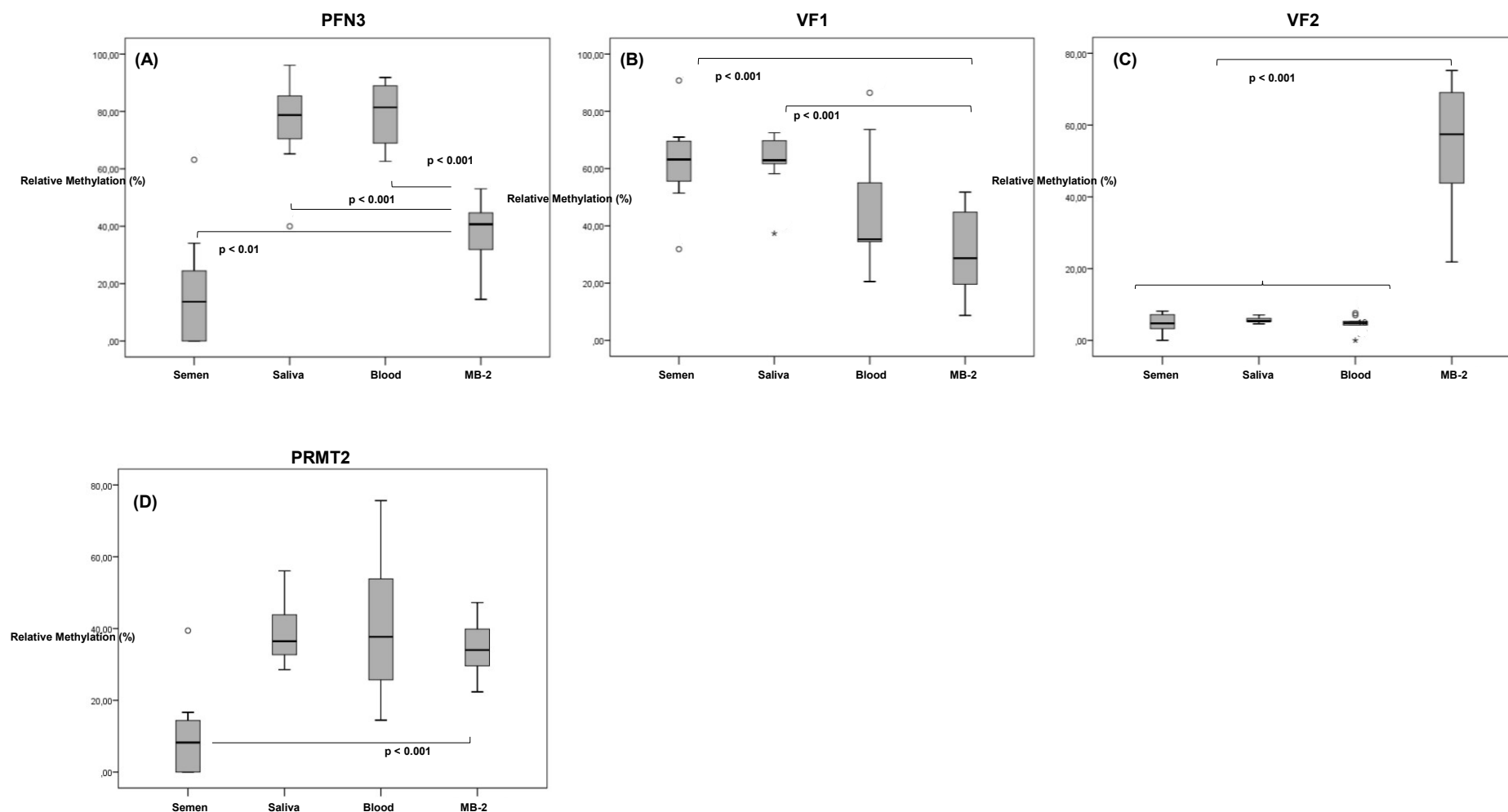


Fig. S1 Relative DNA methylation (%) in the tDMRs for the detection of menstrual blood in the multiplex SNaPshot assay. From left to right semen, saliva, venous blood, and menstrual blood from day 2 of the menses (=MB-2). The methylation state in PFN3 was significantly lower in menstrual blood compared to saliva and venous blood ($p < 0.001$), and significantly higher than in semen ($p < 0.01$) (A). The tDMR for VF1 expressed statistically highly relevant differences in relative methylation between menstrual blood and semen as well as saliva ($p \leq 0.001$). No statistically relevant differences between states of methylation between menstrual blood and venous blood were detected (B). In VF2 menstrual blood expressed significant hypermethylation compared to semen, saliva, and blood ($p < 0.001$) (C). Marker PRMT2 expressed highly statistically significantly greater methylation in menstrual blood than in semen ($p < 0.001$). Relative methylation in menstrual blood was statistically insignificantly different from relative methylation in saliva and venous blood. Circles represent outliers and asterisks represent extreme values.

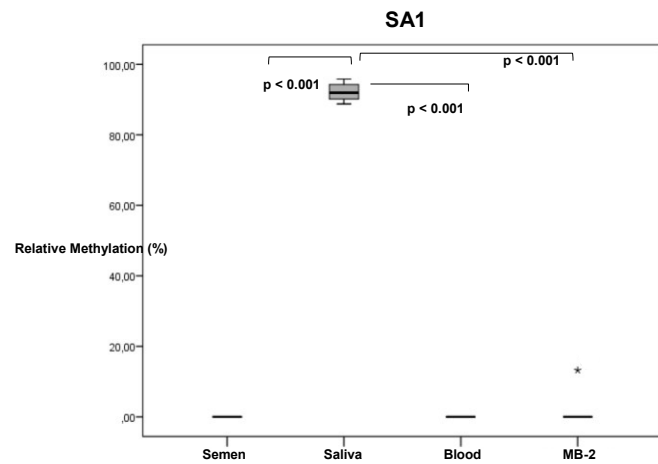


Fig. S2 Relative DNA methylation (%) in the tDMR for the detection of saliva in the multiplex SNaPshot assay. From left to right semen, saliva, venous blood, and menstrual blood from day 2 of the menses (=MB-2). Only in saliva SA1 showed almost complete methylation, while all other body fluids expressed hypomethylation. Differences in relative methylation were highly statistically significant ($p < 0.001$), allowing unambiguous distinguishing between saliva and semen, venous blood, and menstrual blood. Circles represent outliers and asterisks represent extreme values.

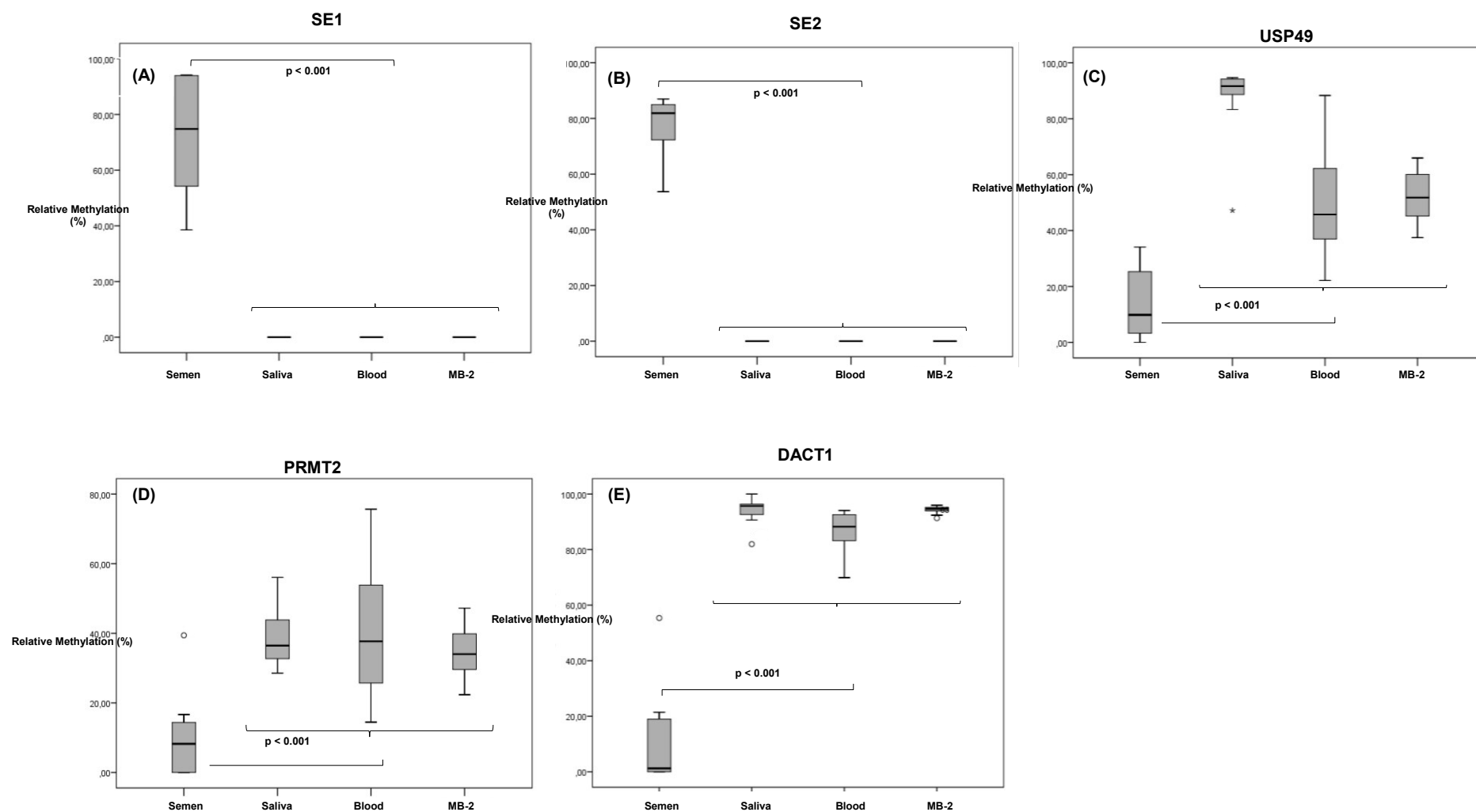


Fig. S3 Relative DNA methylation (%) in the tDMRs for the detection of semen in the multiplex SNaPshot assay. From left to right semen, saliva, venous blood, and menstrual blood from day 2 of the menses (=MB-2). Markers SE1 (A) and SE2 (B) expressed significantly semen-specific hypermethylation ($p < 0.001$). Marker SE2 showed lower intersample variation than SE1. Relative methylation for USP49 (C) was significantly lower in semen compared to saliva, menstrual blood, and venous blood ($p < 0.001$). In PRMT2 (D) relative methylation in semen was significantly lower than in menstrual blood, venous blood, and saliva ($p < 0.001$). In DACT1 (E) semen expressed significantly lower relative methylation than all other body fluids ($p < 0.001$). Circles represent outliers and asterisks represent extreme values.

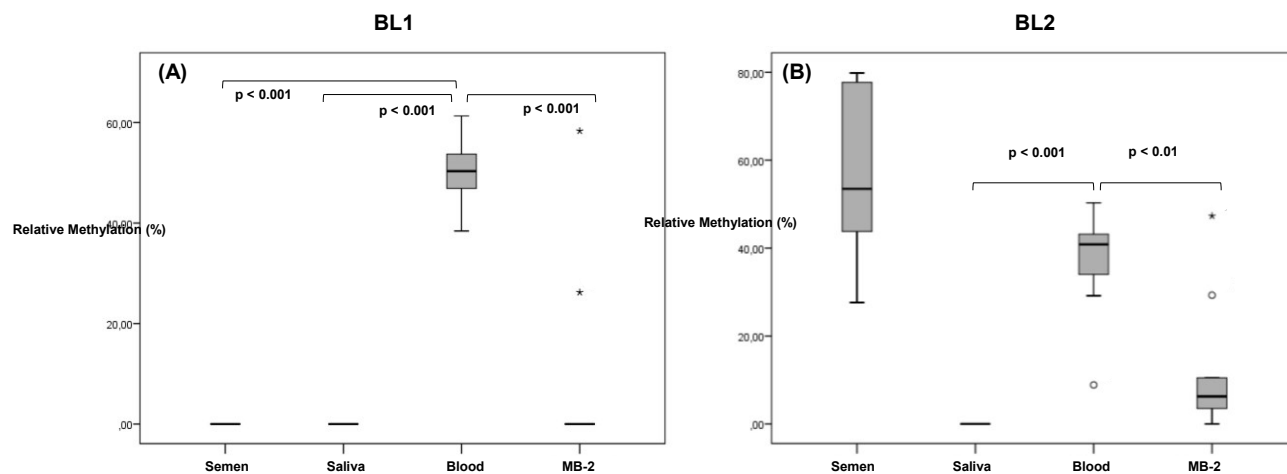


Fig. S4 Relative DNA methylation (%) in the tDMRs for the detection of venous blood in the multiplex SNaPshot assay. From left to right semen, saliva, venous blood, and menstrual blood from day 2 of the menses (=MB-2). Marker BL1 (A) expressed significant hypermethylation in venous blood with low to no methylation in all other body fluids ($p \leq 0.001$). In BL2 (B) saliva samples expressed complete unmethylation. Relative methylation in venous blood was significantly higher than in saliva ($p < 0.001$) and in menstrual blood ($p < 0.01$) but insignificantly lower than in semen. Circles represent outliers and asterisks represent extreme values.

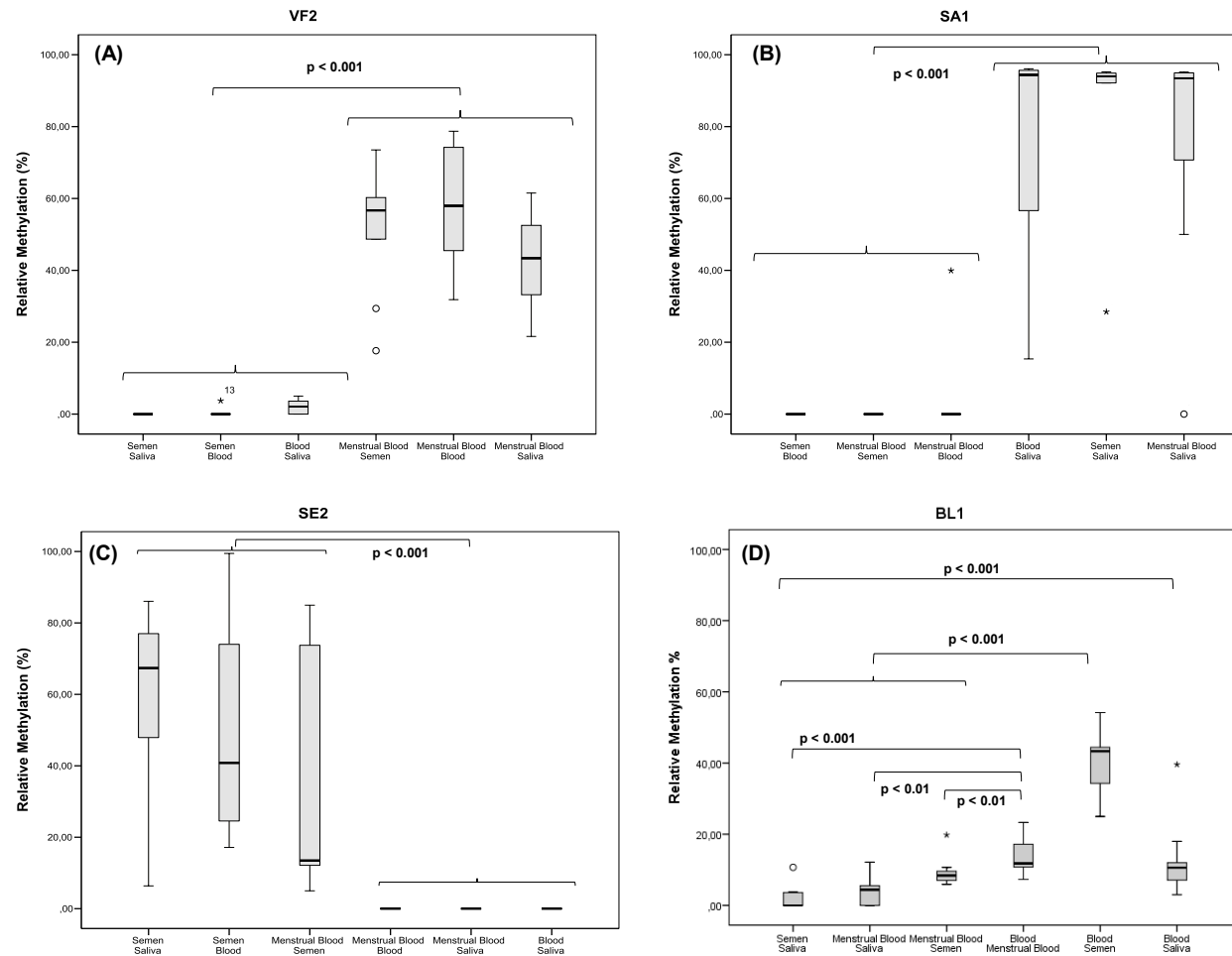


Fig. S5 Relative DNA methylation (%) in the tDMRs of body fluid mixtures in the multiplex SNaPshot assay. In VF2 (A) menstrual blood-containing mixtures expressed a significantly higher methylation state than non-menstrual-blood-containing mixtures ($p < 0.001$). In SA1 (B) the saliva-containing mixtures expressed a significantly higher methylation state than non-saliva-containing mixtures ($p < 0.001$). In SE2 (C) semen-containing mixtures expressed a significantly higher methylation state non-semen-containing mixtures ($p < 0.001$). In BL1 (D) the mixture of venous blood and menstrual blood expressed a significantly greater methylation state than the mixtures of semen and saliva ($p < 0.001$), menstrual blood and saliva ($p < 0.01$), and menstrual blood and semen ($p < 0.01$). The mixture of venous blood and semen also expressed a significantly greater methylation state than the mixtures of semen and saliva, menstrual blood and saliva, and menstrual blood and semen ($p < 0.001$). The mixture of venous blood and saliva expressed a significantly higher methylation state than the mixture of semen and saliva ($p < 0.001$). Circles represent outliers and asterisks represent extreme values.